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Stable expression in mouse cells of nuclear neoantigen after transfer of a 3.4-megadalton cloned fragment of Epstein–Barr virus DNA

(Epstein–Barr nuclear antigen/gene transfer)

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ABSTRACT **All cells that harbor the Epstein–Barr virus (EBV) genome contain a neoantigen in the nucleus (EBNA). By transfection we located a segment of the genome that encodes or induces an antigen serologically related to EBNA. The responsible genes are found in the 3.4-megadalton *Bam*HI fragment K of EBV DNA, specifically in the left 1.9 megadaltons represented by *Hin*dIII fragment I_1. Mouse LTK$^-$ cells were cotransformed with recombinant plasmids containing the herpes simplex virus thymidine kinase gene and either *Eco*RI fragment B or *Bam*HI fragment K of EBV DNA. The TK$^+$ cells surviving in selective medium were cloned. About 50% of the clones expressed the neoantigen in every nucleus. These mouse cells were used as antigens in immunofluorescence tests. Antibody to the nuclear antigen was found in 30 human sera known to contain antibody to EBNA; it was not detected in 18 sera that did not have antibody to EBNA. Mouse cells expressing EBNA as the result of acquisition of cloned EBV DNA fragments should prove useful in the characterization of the structure of this antigen and as reagents for the diagnosis of EBV infections.**

Genetic study of the human lymphotropic herpes virus Epstein–Barr virus (EBV) is hindered by the lack of mutants and by the absence of a fully permissive host cell that allows genetic recombination between viruses. In view of these limitations, a suitable approach for defining the products of some EBV genes is gene transfer with defined segments of viral DNA. This approach is feasible because DNA prepared from virions is infectious by either microinjection or transfection (1–3). Human placental fibroblasts exposed to intact virion DNA produce EBV which is able to immortalize lymphocytes (ref. 2; unpublished data). Several different types of tissue culture cells exposed to EBV DNA display various morphologic forms of antigens in the cytoplasm, nuclear membrane, and nucleus; these antigens are detectable by certain human sera with antibody to EBV (3, 4).

If a mixture of virion DNA fragments produced by cleavage with one of several restriction endonucleases is introduced, antigens also appear in the fibroblasts. If *Eco*RI and *Sal* I are used, antigens appear in both nucleus and cytoplasm but if *Bam*HI and *Hin*dIII, are used, the antigen seems to be limited to the nucleus (4). The need to identify this nuclear antigen and its relationship to "EB nuclear antigen" (EBNA) underlies the present experiments.

The finding that a mixture of virion DNA fragments led to nuclear antigen expression suggested the possibility that individual cloned subfragments of viral DNA would also be able to induce the antigen. We found that a single large cloned fragment, *Eco*RI fragment B of EBV (FF41) DNA, approximately 19 megadaltons, was competent to cause expression of a nuclear antigen in human fibroblasts (4). We were unable to study this nuclear antigen in human cells by anticomplement immunofluorescence, the usual immunologic assay for EBNA, because of nonspecific binding of complement to the cytoskeleton of the placental fibroblasts. Furthermore, in this nonselective transfection system the nuclear antigen appeared transiently and was limited to a small fraction of cells (about 0.1% or less).

We have now introduced EBV *Eco*RI fragment B and subclones of this region under selective conditions that would favor the retention and possible expression of the EBV genes in a high fraction of surviving cells. The selection system was cotransformation of mouse LTK$^-$ cells with the herpes simplex virus (HSV) thymidine kinase (TK) gene (*tk*) and cloned pieces of EBV DNA. Most of those cells that acquire the DNA encoding the herpes viral enzyme also pick up other DNA fragments present in the transfection mixture (5). We find that the EB nuclear neoantigen is stably expressed in mouse cells after introduction of a relatively small EBV DNA fragment.

MATERIALS AND METHODS

Cells. LTK$^-$ aprt$^-$ cells originally derived from the LTK$^-$ line (6) were obtained from Saul Silverstein. These cells are maintained by weekly 1:25 splits.

DNA. The herpes *tk* gene was in the form of a *Bam*HI fragment of HSV-1 DNA cloned in pBR322. This plasmid, designated pXI, was obtained from a "cleared lysate" and the DNA was purified on a CsCl gradient (7). EBV (FF41) *Eco*RI fragment B was cloned in pACYC 184; the plasmid is designated p*Eco*B; five DNA fragments which are contained partially or wholly within p*Eco*B—namely, *Hin*dIII E_f and *Bam*HI K, R, B, and G—were cloned in pBR322 (Fig. 1). All the EBV DNA clones were purified on sucrose gradients (4). The physical map of the *Bam*HI fragment K clone was prepared by means of double digestion of the DNA with different restriction endonucleases and by hybridization of Southern blots with *Hin*dIII subfragment I_1 of *Bam*HI fragment K.

Transformation. The calcium phosphate coprecipitation technique was used (9). Carrier DNA prepared from LTK$^-$ cells was present at a final concentration of 20 μg/ml; the concentrations of HSV *tk* and EBV DNAs were varied. Carrier and plasmid DNAs were combined in the appropriate ratios in 250 mM $CaCl_2$ and then dripped into an equal volume of Hepes PO_4 buffer, exactly as described by Wigler *et al.* (10). The precipi-

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Abbreviations: EBV, Epstein–Barr virus; TK and *tk*, thymidine kinase enzyme and gene, respectively; HSV, herpes simplex virus; EBNA, Epstein–Barr nuclear antigen.

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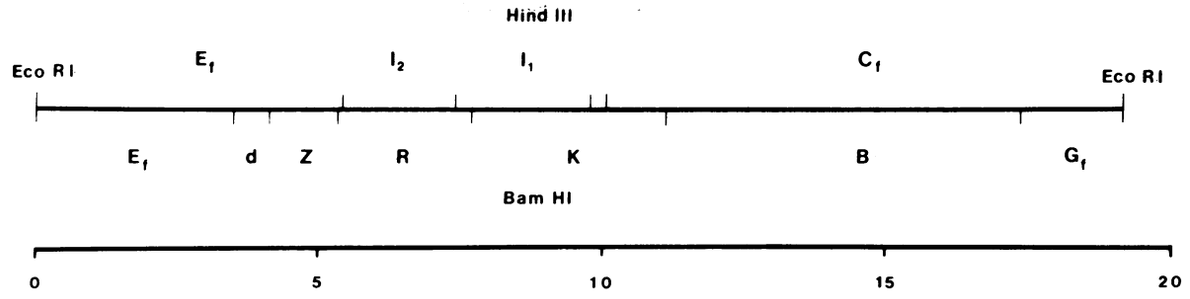

FIG. 1. Physical map of *Eco*RI fragment B of EBV DNA, strain FF41 (8). Size scale is in megadaltons.

tates were held at room temperature for 0.5 hr and then 0.4-ml samples were added to LTK$^-$ cells in 60-mm plates. After 6 hr at 37°C, the precipitates were removed and nonselective medium was added for overnight incubation. Selective medium (0.6 μM methotrexate/50 μM adenosine/50 μM guanosine/0.1 mM glycine/16 μM thymidine) was introduced the following day (11). This medium was replaced every 3 days. Colonies that survived selection were counted after 14 days.

Cell Clones. All transformed colonies from a single dish were initially combined. After antigen expression was evident, the cells were cloned in microtiter plates by limiting dilution. Individual wells contained an estimated 2, 1, or 0.5 cell per well. When a single colony was present the cells were dispersed with trypsin and propagated.

Immunofluorescence Assays. The source of antigen was LTK$^-$ cells cotransformed with HSV *tk* and either the *Eco*RI B or *Bam*HI K fragment of EBV. Antigen controls were LTK$^-$ cells with HSV *tk* alone. Cells grown on coverslips were fixed 3–5 days after seeding. Two types of assays were performed, indirect anti-immunoglobulin (anti-Ig) and indirect anti-C immunofluorescence. For the former test, cells were fixed in acetone; for the latter, fixation was in methanol. The presence of antibody to early antigen in human sera was determined by the indirect anti-Ig method on Raji cells that had been superinfected with the HR-1 strain of EBV (12). Antibody to EBNA in these sera was measured by the anti-C method on Raji cells (13). For initial screening of transfected cells we used a 1:40 dilution of a "standard" human serum with high titers of anti-EBNA and anti-early antigen as described (4). As a control we used a 1:10 dilution of a human serum lacking EBV antibody. In subsequent tests we used a panel of different human sera with and without antibody to EBNA and early antigen. These sera were all screened at a 1:10 dilution. Conjugates, purchased commercially, were fluorescein isothiocyanate-conjugated sheep anti-human Ig and either fluorescein- or rhodamine-conjugated goat anti-human C. All conjugates were used at a dilution of 1:30. Diluent for sera was phosphate-buffered saline in the anti-Ig test and gelatin/Veronal buffer in the anti-C test.

RESULTS

Antigen Expression in LTK$^-$ Cells Cotransformed with HSV *tk* and Plasmids Containing the EBV *Eco*RI Fragment B and *Bam*HI Fragment K. In our first cotransformation experiments we used *Eco*RI fragment B of EBV DNA because this fragment was known to induce antigens in Vero and human placental cells (4) and because this region of DNA encodes a large number of viral mRNAs in cells producing viral DNA and viral antigens (unpublished data). Accordingly, p*Eco*B was introduced into LTK$^-$ cells with the HSV *tk* gene and selection was applied for cells that could express the viral enzyme (Table 1). p*Eco*B, by itself, did not allow the LTK$^-$ cells to grow in selective medium, a finding that implied that this fragment of EBV DNA did not encode a thymidine kinase. Colonies were fewer in number when the HSV *tk* was cotransferred in combination with p*Eco*B than when the *tk* gene was used alone. This result suggested that p*Eco*B might encode a cytotoxic function.

A pool of *tk*-transformed cells from cultures that had received the plasmids with *Eco*RI fragment B and HSV *tk* was examined for antigen, with the standard EBV-positive serum, by anti-Ig and anti-C immunofluorescence. Between 3% and 15% of surviving cells that had received the EBV DNA clone (group 7, Table 1) contained antigen in the nucleus. This antigen was not seen when the cells from the same culture were treated with three human sera lacking EBV antibodies. Nor were antigens found in pooled transformed cells that had received only HSV *tk* (group 3, Table 1).

The sequences in the 19.1-megadalton *Eco*RI fragment B are encompassed by four subfragments produced by digestion with *Bam*HI (fragments R, K, B, and G in Fig. 1) and by *Hin*dIII fragment E, the left terminus. Recombinant DNA clones containing these five fragments were used individually and in combination in further cotransformation experiments (Table 2). In two replicate experiments, antigens were sought in cells from a pool of *tk*-transformed colonies that arose after exposure to the subfragments of p*Eco*B. Nuclear antigen was seen only in cells that had received HSV *tk* and the *Bam*HI fragment K of EBV (FF41) DNA either alone or as part of a mixture of EBV DNA clones. In these cotransformations, 30–70% of the original population of cells expressed antigen. The other *Bam*HI fragments located in *Eco*RI fragment B did not induce neoantigens, nor did *Hin*dIII fragment E.

Antigen Expression in Cell Clones of LTK$^-$ Cells Transformed by *Eco*RI Fragment B or *Bam*HI Fragment K. Only a fraction of cells derived from a mixture of colonies that received either *Eco*RI fragment B or *Bam*HI fragment K of EBV DNA expressed nuclear antigen. Was this the result of intermittent antigen expression by cells, or did the transformed cells consist of a mixture, some that were antigen positive and others

Table 1. Cotransformation of LTK$^-$ cells with HSV *tk* (pXI) and EBV p*Eco*B

Group	Contents	Colonies, no./dish*	Cells with antigen, %†	
			Anti-C	Anti-Ig
1	$CaPO_4$	0	—	—
2	Carrier only	0.3	ND	ND
3	pXI (0.1 μg)	146	0	0
4	pXI (0.03 μg)	40	ND	ND
5	p*Eco*B (1.0 μg)	0	—	—
6	p*Eco*B (0.3 μg)	0	—	—
7	p*Eco*B (1.0 μg)/pXI (0.1 μg)	36	9	13
8	p*Eco*B (0.3 μg)/pXI (0.03 μg)	26	5	5

* Mean of three dishes in one experiment.
† Standard EBV-seropositive human serum was used. ND, not done.

Table 2. Cotransformation of LTK$^-$ cells with a cloned HSV *tk* gene and subfragments of EBV (FF41) *Eco*RI fragment B

Group	Contents	Colonies, no./dish*	Cells with antigen, %†	
			Anti-C	Anti-Ig
1	Carrier only	0	—	—
2	pXI‡	27	0	0
3	pXI + p*Bam*HI R§	27	0	0
4	pXI + p*Bam*HI K§	33	69	46
5	pXI + p*Bam*HI B§	44	0	0
6	pXI + p*Bam*HI G§	24	0	0
7	pXI + p*Hin*dIII E§	28	0	0
8	pXI + mixture 3-7¶	14	66	31
9	pXI + p*Eco*B	5	ND	ND

* Mean of three or four dishes in two experiments.
† Standard EBV-seropositive human serum used. ND, not done.
‡ At 0.1 μg per dish in groups 2–9.
§ At 1.0 μg per dish.
¶ Mixture contained 1.0 μg of each EBV DNA fragment.

that were antigen negative? To answer this question, a set of 49 single-cell clones was obtained from group 7 (Table 1) which had been derived by pooling of colonies transformed by HSV *tk* in the presence of EBV p*Eco*B. The cloning efficiency was nearly 100%. Twenty-three of the 49 clones contained antigen detectable by anti-C immunofluorescence in all the nuclei. With the standard human serum the same 23 clones also contained a nuclear antigen detectable by indirect anti-Ig immunofluorescence. The remaining 26 clones were antigen negative.

In those clones positive by the anti-C test, every nucleus contained the antigen (Fig. 2 *Left*). By contrast, the extent of expression of antigen detectable by anti-Ig differed widely among the clones. In seven clones, 100% of the cells were positive by anti-Ig; in three clones, about 50% of the nuclei were positive. In 13 clones the fraction of nuclei positive for antigen by anti-Ig was low, from 8% of cells positive to only a rare cell. With both tests there was always variation in the intensity of antigen expression from cell to cell even in clones in which the majority of cells contained antigen (Fig. 2 *Right*).

We also derived cell clones from the mixed population of LTK$^-$ cells that expressed nuclear antigen after cotransformation by HSV *tk* and EBV *Bam*HI fragment K (group 4, Table 2). Three of nine clones were antigen positive; six were negative. In the three positive clones, antigen was found by both anti-Ig and anti-C in all the cells.

Stability of Antigen Expression in Cotransformed LTK$^-$ Cells. Antigen detectable by the anti-C test has been stably expressed for at least 16 weekly passages of the original LTK$^-$ cells exposed to the *Eco*RI fragment B. Cells containing *Bam*HI fragment K have expressed antigen in >70% of nuclei for 10 consecutive passages. Although the derivative cell clones have been available for a shorter time, antigen has remained stable in them too. However, expression of antigen revealed by anti-Ig has been more variable. One cell clone which originally contained antigen in every nucleus by anti-Ig eventually changed so that antigen was only found in an occasional nucleus.

Transient Expression of Antigen in LTK$^-$ Cells. EBV antigens were also expressed in mouse LTK$^-$ cells when DNA was introduced without the HSV *tk* selection system. Antigens were evident as early as 24 hr after transfection but reached a maximum after 48–72 hr. Intact viral DNA induced about 2,500

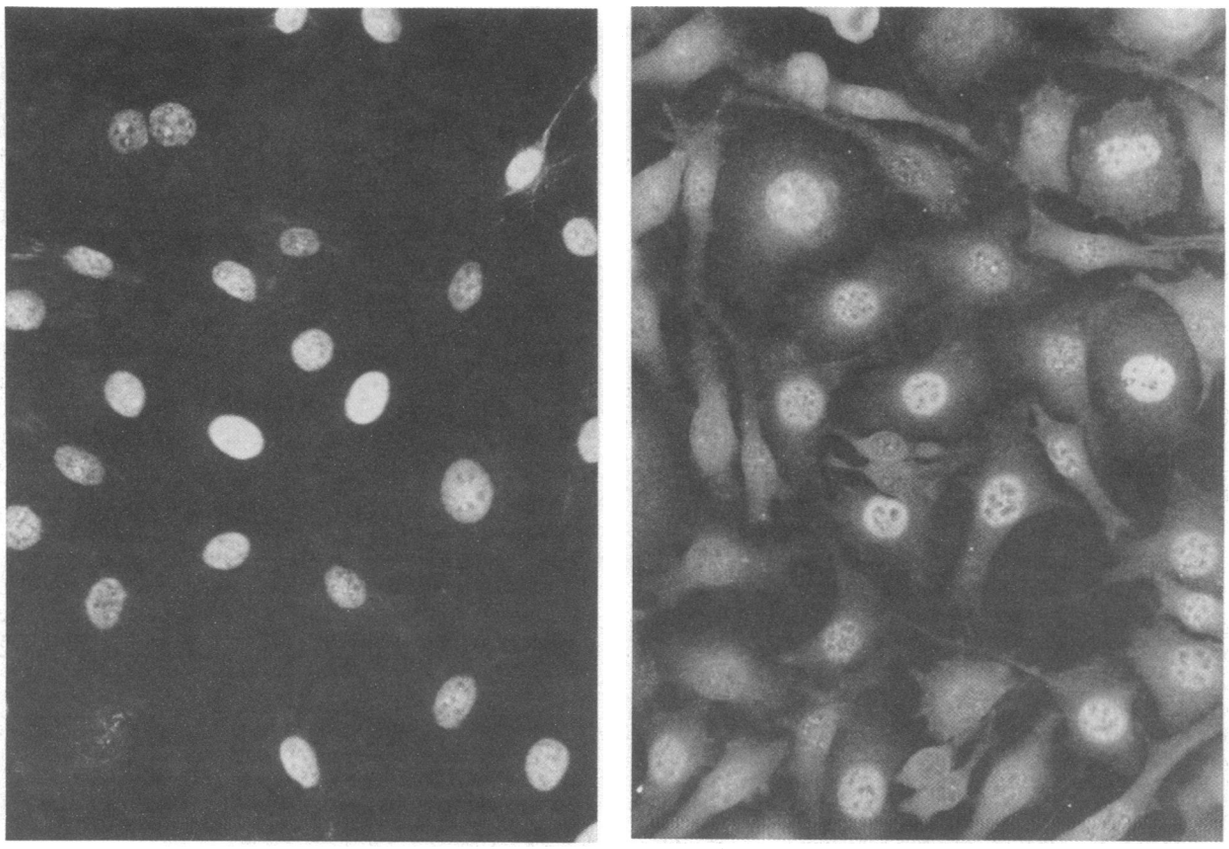

FIG. 2. Antigen expression in the H_1 clone of LTK$^-$ cells transformed by EBV *Eco*RI fragment B. (*Left*) Anti-C immunofluorescence with a rhodamine-conjugated anti-human C_3. (*Right*) Anti-Ig immunofluorescence with fluorescein-conjugated anti-human Ig. The source of antibody in both tests was serum from a Chinese patient with nasopharyngeal carcinoma.

Table 3. Antigen expression in LTK⁻ cells exposed to *Bam*HI fragment K of EBV DNA digested with different restriction endonucleases

Enzyme	Subfragments, no.	Sizes, megadaltons	Antigen-positive cells, no./coverslip* Anti-C	Anti-Ig
None	0		546	177
Sal I	0		311	26
Bgl II	0		169	32
Xba I	2	2.05; 1.2	496	47
*Hinc*II	3	1.75; 1.45; 0.19	69	6
*Hind*III	3	1.9; 1.14; 0.34	47	5
*Hinf*I	≥6		0	0
Alu I	≥6		0	0

* Coverslips were fixed 96 hr after transfection with 2.4 μg of plasmid DNA containing *Bam*HI fragment K, undigested or digested with the indicated endonuclease.

Table 4. Reactivity of human sera with nuclear neoantigen in LTK⁻ cells containing EBV *Bam*HI fragment K correlates with presence of antibody to EBNA

Group	Antibody status of serum* Anti-VCA	Anti-EA	Anti-EBNA	Sera tested, no.	Positive on LTK⁻*Bam*HI K, no.†
1	−	−	−	7	0
2	+	−	−	2	0
3	+	+	−	9	0
4	+	−	+	16‡	15
5	+	+	+	19§	15

* VCA, viral capsid antigen; EA, early antigen.
† The uncloned mixed population (group 4, Table 2) with 70% antigen-positive cells was used as the antigen. Both anti-Ig and anti-C tests were used.
‡ The single nonreactive serum had an anti-EBNA titer of 1:10.
§ The anti-EBNA titers of the four nonreactive sera were 1:2, 1:2, 1:5, and 1:10.

antigen-positive cells per μg, whereas the plasmid containing *Eco*RI fragment B had a specific infectivity of about 800 antigen-positive nuclei per μg of DNA. In several repeated experiments the specific infectivity of cloned *Eco*RI fragment B was 10–30% that of virion DNA assayed in parallel. After exposure to intact DNA or *Eco*RI fragment B under nonselective conditions, LTK⁻ cells displayed antigens in the cytoplasm and in the nucleus that were similar morphologically to the antigens described in human fibroblasts or monkey kidney lines (4).

Permanent cotransformation was not required for expression of EBV antigens in L cells exposed to *Bam*HI fragment K; however, after treatment with this piece of DNA, the antigens were seen only in the nucleus. To delineate further the coding region for nuclear antigen the plasmid containing *Bam*HI fragment K was digested with seven restriction endonucleases. The mixture of fragments resulting from digestion with each enzyme was introduced into LTK⁻ cells under nonselective conditions and antigens were sought after 96 hr (Table 3; Fig. 3). Antigen expression was not destroyed by two enzymes, *Sal* I and *Bgl* II, which do not cut *Bam*HI fragment K; by contrast, *Alu* I and *Hinf*I, which digest *Bam*HI fragment K into six or more subfragments, interrupted the responsible genes. Antigen expression remained intact when *Xba* I, which has only one site in *Bam*HI fragment K, was used. A mixture of *Hind*III or *Hinc*II fragments also induced the nuclear antigen, but in fewer cells.

The left end of *Bam*HI fragment K is colinear with a *Hind*III fragment designated I_1 (Fig. 3). We found that recombinant plasmid pBR322 containing *Hind*III fragment I_1, with a size of 2.2 megadaltons, induces the nuclear antigen. After exposure of LTK⁻ cells to about 3 μg of this plasmid, there were more than 1,300 nuclear-antigen-positive cells per coverslip by anti-C and 250 cells with antigen detectable by anti-Ig. Therefore, the genetic information for induction of this antigen resides on the leftmost 1.9 megadaltons of *Bam*HI fragment K.

To learn whether any other EBV DNA fragment located outside the *Eco*RI fragment B region was able to cause antigens to appear in the LTK⁻ cells, we used recombinant pACYC 184 plasmids containing *Eco*RI fragments C, E, F, G_1, H, I, and J of EBV (FF41) DNA (4) and two clones of EBV (B95-8) DNA obtained from J. Arrand (*Eco*RI fragment A and "terminal") (14). When introduced under nonselective conditions, none of these other *Eco*RI EBV DNA fragments caused antigen detectable by immunofluorescence to appear.

To complete the survey of the entire EBV genome we introduced *Bam*HI fragments O, M, L, and E, which cover regions not included in this set of *Eco*RI fragments [see ref. 8 for a map of EBV (FF41) fragments]. A few antigen-positive nuclei were seen after transfection with *Bam*HI fragment M.

Reactivity of the Nuclear Antigen with Human Sera. To learn whether the nuclear antigen found in the mouse LTK⁻ cells corresponded to any known EBV antigen that had been described in lymphoid cells, a panel of 53 different sera was studied. These sera, selected to represent a spectrum of reactivity to different EBV antigens, were obtained through the courtesy of W. A. Andiman and W. Henle and were from patients with various clinical syndromes associated with EBV such as infectious mononucleosis, pneumonia, thrombocytopenia, and diffuse lymphoma as well as from patients with unrelated diseases. The sera were coded and tested, by both anti-C and anti-Ig, on antigen consisting of LTK⁻ cells cotransformed by HSV *tk* and *Bam*HI fragment K of EBV DNA (Table 4).

There was a strong correlation between antibody to EBNA and the reactivity with the nuclear antigen in L cells containing *Bam*HI fragment K. Thirty of 35 anti-EBNA-positive sera detected nuclear antigen in the mouse cells. There were low titers (1:10 or lower) of antibody to EBNA in the five anti-EBNA-positive sera that failed to react with the L cells. The nuclear antigen in the mouse cells was detectable with all (22/22) sera with an anti-EBNA titer of 1:20 or greater. Six of eight sera with

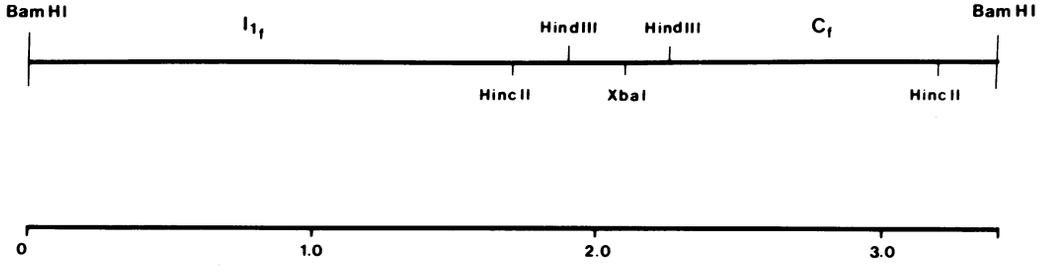

FIG. 3. Physical map of *Bam*HI fragment K of EBV DNA, strain FF41. Size scale is in megadaltons.

a titer of 1:10 and two of three sera with a titer of 1:5 were also positive. With reactive sera the antigen could be found either by anti-Ig or by anti-C immunofluorescence.

DISCUSSION

By means of transfection experiments with cloned fragments of Epstein–Barr viral DNA we have located genes that encode or induce one or more nuclear neoantigens. Because the responsible segment of DNA represents less than 2% of the genome, this should simplify the analysis of the molecular genetics of this neoantigen and permit determination of the nucleotide sequence of the responsible genes, a clear definition of the structure of the antigen, and, hopefully, an understanding of its function in the process of lymphocyte immortalization. Not only does the responsible DNA fragment call forth the synthesis of the antigen when it is introduced in the selective *tk* cotransformation system but also the antigen appears within 24 hr after the fragment is provided to the cells under nonselective conditions. Therefore the *Bam*HI K fragment of EBV DNA is likely to contain one or more strong promoters of transcription.

At least one component of the nuclear neoantigen detected in mouse cells is serologically related to EBNA (13). EBNA is thought to be an important product of the EBV transformation process. It seems to bind to chromosomes and to DNA (15, 16). EBNA is invariably expressed in cells that harbor EBV DNA, and its synthesis is the earliest event identified thus far in the process of lymphocyte immortalization (17, 18). Among viral mRNA transcripts found in cells immortalized *in vitro* or in Raji cells is one from *Bam*HI fragment K (19, 20). Because these cells do not produce virions, it has been thought that this messenger represents a viral product that is synthesized in transformed cells. Our results imply that EBNA is a product of *Bam*HI fragment K mRNA.

The evidence that the nuclear neoantigen corresponds to EBNA rests on the immunologic assays (Table 4). The nuclear antigen in L cells is reactive with nearly all human sera that contain antibody to EBNA in Raji cells, and the antigen fails to react with human sera that lack anti-EBNA activity. The morphology of the antigen resembles that seen in Raji cells, and the antigen is revealed by the anti-C test usually used to detect EBNA. In contrast to EBNA found in lymphoid cells, the nuclear neoantigen seen after cotransformation of mouse cells is also detectable by anti-Ig immunofluorescence, albeit in fewer cells and with some irregularity from cell to cell. In those cells in which large amounts of the antigen are made, or perhaps in which the antigen is not all bound to chromatin, the anti-Ig test may be sensitive enough to detect the antigen. Because the exact structure of EBNA is not known and there are no monospecific antibodies to EBNA, it is not possible to conclude that the nuclear neoantigen found in transfected mouse cells and EBNA present in lymphoid cells are identical. This conclusion must rest ultimately on a biochemical definition of the antigens.

Heretofore, EBNA has not been detected in a nonlymphoid cell exposed *in vitro* to EBV DNA by transfection or by microinjection (1–3), and only a rare EBNA-positive cell was found in mouse 3T3 cells infected by intact EBV virions by the receptor implantation technique (21). These negative results had raised the possibility that EBNA might not be expressed in all types of differentiated cells. However, our findings make it likely that EBNA can be expressed in various cell types once the responsible genes are appropriately introduced.

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